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13. ABSTRACT *(Maximum 200 Words)*

The failures to growth arrest in response to critically short telomeres or to TGFβ are key derangements thought to contribute to the inappropriate cell growth that characterizes breast cancer progression. Our recent observations indicate that activation of telomerase activity may be directly involved in overcoming both inhibitory pathways in human mammary epithelial cells (HMEC). By exploring the common thread connecting telomerase expression and resistance to TGFβ growth inhibition, it may be possible to unify two divergent areas of significance for breast cancer development and treatment. We have begun to dissect the complex mechanisms responsible for these changes in expectation that such knowledge may enable us to prevent or reverse them. Our work thus far indicates that altered regulation of a well-known oncogene, c-myc, may link these processes by lowering the external signaling requirements for cell proliferation. It is not yet clear, however, how hTERT expression leads to altered c-myc regulation. The ability of the hTERT protein to cause TGFβ resistance does not appear to be separable from its ability to bind and lengthen telomeres in vivo. The ability of hTERT to cause TGFβ resistance depends on cell context; only HMEC lacking p16 expression were susceptible to hTERT immortalization and TGFβ resistance. This suggests that particular cell types may be targeted in vivo.

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INTRODUCTION

The failures to growth arrest in response to critically short telomeres or TGFβ are key derangements contributing to the inappropriate cell growth that characterizes breast cancer progression. Our recent observations indicate that activation of telomerase activity may be directly involved in overcoming both inhibitory pathways in human mammary epithelial cells (HMEC). By exploring the common thread connecting telomerase expression and resistance to TGFβ growth inhibition, it may be possible to unify two divergent areas of significance for breast cancer development and treatment.

BODY

(Note: Because of the significant time that elapsed between when the proposal for this work was submitted (6/1/1999) and when the proposal was actually funded (8/7/2000), additional preliminary data was gathered during the interim. These results, as they pertain to Specific Aims, are presented here. Because of the ambitious nature of the work proposed, we do not anticipate that any changes in the Specific Aims of the project will be required to compensate for the work already completed.)

Technical Objective 1: Determine whether reduced p27 levels alone can interfere with TGFβ-mediated growth inhibition of HMEC.

We learned at the June, 2000 DOD Era of Hope meeting in Atlanta that the p27 blocking experiment had already been performed by Joyce Slingerland of our collaborating group at the U.Toronto. The results indicated that antisense p27 expression was incapable of interfering with TGFβ-mediated growth inhibition in normal finite lifespan HMEC. In light of this development, we refocused our attention on different mechanisms that might mediate the growth inhibition. Another important aspect of the TGFβ anti-proliferative program is the inhibition of c-myc expression. C-myc is a ubiquitous transcription factor involved in cell proliferation. Levels of c-myc mRNA and protein in normal HMEC are decreased in response to TGFβ. Massague and co-workers have recently demonstrated that TGFβ rapidly induces the formation of a protein complex that specifically recognizes an inhibitory element in the c-myc promoter (1). Formation of the complex is deficient in oncogenically transformed breast cells, suggesting that the complex that mediates c-myc repression is a target of oncogenic signals. Consistent with this hypothesis, we find that fully immortal HMEC exhibit deregulated c-myc (Fig.1). Although the differences in cycling cells are marginal and could arguably be due to differences in proliferation rate, the differences in cells synchronized in G0 are striking. Another recent development ties hTERT expression to c-myc deregulation. Beach and co-workers showed that when an exogenously introduced hTERT gene was excised from normal HMEC, c-myc protein levels were 2-3 fold higher, and the cells continued to express endogenous telomerase (2). In our lab, we have also examined c-myc expression in hTERT-infected HMEC. When we compared c-myc protein in hTERT-infected HMEC to control HMEC with similar labeling indices, we first saw increased c-myc protein in cycling cells, but not in cells arrested in G0 (Fig.2). However, a later passage of hTERT-infected cells showed increased myc in both G0 and cycling cells. The other striking finding in this experiment was that the hTERT-infected HMEC no longer growth-arrested as well in the absence of EGF receptor signal transduction. Note that the labeling index in the hTERT-transduced HMEC was ~ 30% vs. 6% in controls. Based on our results and those of Beach's group, we hypothesized that differences in amplification of the c-myc locus could be responsible for differences in the abilities to grow in the presence of TGFβ. However our preliminary comparative genomic hybridization (CGH) data indicates that

overexpression of hTERT is not associated with c-myc amplification or general genomic instability in our normal HMEC (data not shown). An alternative hypothesis is that hTERT and myc may compose a positive feedback loop in which one reinforces the expression of the other over time. We are currently confirming our preliminary results, and examining possible mechanisms.

Technical Objective 2: Determine how TGFβ-mediated stabilization of p27 is disrupted in hTERT expressing cells.

These experiments have not yet been started (See SOW).

Technical Objective 3: Determine whether hTERT-induced resistance to TGFβ-mediated growth inhibition requires continuous telomerase activity and/or access to telomeres.

To determine what functions of the ectopic hTERT are required for induction of TGFβ resistance, two different hTERT mutants were introduced into 184 HMEC at 12p. One mutant has inactivating amino acid substitutions in the reverse transcriptase domain (3), and the other is a wild type hTERT with a carboxyl-terminal HA epitope tag that shows *in vitro*, but not *in vivo*, telomerase activity (4). These constructs induced either no or very low levels of TGFβ resistance (Table 1), suggesting that the reverse transcriptase domain must be intact and hTERT must be capable of forming a complex with *in vivo* activity to confer TGFβ resistance.

Table 1: Colony growth of post-selection HMEC ±hTERT and ±TGFβ

	LABELING INDEX (%)								
Passage Cell	TGFβ (-)				TGFβ (+)				CFE(%)
184(12p)*	<10	10-25	26-50	>50	<10	10-25	26-50	>50	
13 Babe	41	39	19	1	100	0	0	0	8.8
hTERTmut	25	10	40	25	100	0	0	0	4.4
hTERT-HA	27	27	37	9	88	5	3	4	6.2
hTERT	0	0	0	100	0	0	0	100	18.0

Single cells were seeded and the labeling index ±TGFβ in colonies of >50 cells determined.

*: number in parentheses indicates passage level of cell transduction with hTERT or empty vector (Babe).

To determine whether hTERT-induced TGFβ resistance requires continuous access of telomerase to telomeric DNA, we transduced conditionally immortal 184A1 HMEC with genes encoding telomere interacting proteins, TRF1 (5) and TIN2 (6). Long-term overexpression of TRF1 and TIN2 in telomerase(+) tumor cells has been reported to result in gradual and progressive telomere shortening, presumably by binding to telomeres and limiting telomerase access. Preliminary results indicate that TIN2, but not TRF1, overexpression inhibits the acquisition of TGFβ resistance which normally occurs in the conditionally immortal HMEC as they convert to the telomerase(+) phenotype (Fig.3). Although the TIN2 results need to be repeated, they are consistent with the hypothesis that hTERT-induced resistance to TGFβ-mediated growth inhibition requires telomerase access to telomeres.

As described above for Technical Objective 1, we hypothesized that differences in amplification of the c-myc locus could be responsible for the major differences in the abilities of HMEC to grow in the presence of TGFβ. We analyzed the 184A1 cells transduced with control, TRF1, and TIN2 viruses for changes in gene copy numbers. However, our CGH data indicates that although the 184A1 series does show c-myc amplification, the level of amplification does not correlate with TGFβ sensitivity (data not shown).

Technical Objective 4: Determine whether hTERT induces resistance to TGFβ-mediated growth inhibition in other HMEC types.

Most finite lifespan HMEC grown in either MCBD 170 or MM medium cease proliferation after 15-25 PD with high levels of p16 expression (7). To examine the effect of hTERT in these pre-selection HMEC, 184 HMEC growing in MM were transduced at 3p (~18 PD) with the hTERT gene or control LXSN. All hTERT-transduced cultures showed telomerase activity when assayed at 4p; control cultures had no detectable activity (data not shown). Typical for MM-grown HMEC, control cultures maintained heterogeneous growth through passage 4, with areas of small actively growing cells interspersed among non-proliferative cells with a flattened, vacuolated morphology. LXSN-184(3p) exhibited complete growth arrest by 5p. hTERT-184(3p) initially showed growth and morphologies similar to control cells. Of four independently infected dishes, three ceased proliferation by 5p. hTERT-184(3p) from one dish has maintained growth through passage 41. These hTERT-184(3p) HMEC were examined for growth capacity ±TGFβ, p16 expression, telomerase activity, and TRF length. Similar to hTERT-transduced post-selection and 184A1 HMEC, the mean TRF length of hTERT-184(3p) cultures quickly elongated to 10-12 kb (data not shown). There was a gradual decrease in overall p16 protein levels from 4p to 29p, corresponding to a decreasing number of p16(+) cells (Fig. 4A,B). p16(+) cells had the large flat morphology of senescent HMEC, whereas the p16(-) cells had the small refractile morphology characteristic of proliferating HMEC. hTERT-184(3p) was assayed for growth in mass cultures ±TGFβ from 7p to 40p (Fig. 4C). Cultures at 17p and 20p were also assayed to detect rare TGFβ resistant cells. From 7p to 20p, increasing time in TGFβ resulted in severe growth inhibition, with <10% LI after 9 days in TGFβ compared to 40-65% LI in the non-TGFβ-exposed cultures. However, by 20p rare cells capable of very slow growth in TGFβ could be detected. This first indication of continuous growth capacity in TGFβ corresponded to the passage level where most cells no longer expressed p16. Thereafter, the population showed an increasing number of cells capable of progressively better growth in TGFβ. Thus, hTERT did not immortalize nor did it induce TGFβ resistance in the majority of p16(+) HMEC. However, HMEC lacking p16 expression were susceptible to hTERT immortalization and TGFβ resistance.

KEY RESEARCH ACCOMPLISHMENTS

- The c-myc oncogene shows altered regulation in hTERT-transduced HMEC.

- hTERT-transduced HMEC show reduced dependence on EGF receptor mediated signal transduction for growth.

- The hTERT reverse transcriptase domain must be intact and hTERT must be capable of forming a complex with *in vivo* activity in order to confer TGFβ resistance.

• Overexpression of telomere-associated protein TIN2, but not TRF1, inhibited the acquisition of TGFβ resistance in conditionally immortal HMEC as they converted to the telomerase(+) phenotype.

• hTERT did not immortalize nor did it induce TGFβ resistance in the majority of p16(+) HMEC.

REPORTABLE OUTCOMES

Presentation – "Telomere Dynamics and the Development of TGFbeta Resistance during Transformation of Cultured Human Mammary Epithelial Cells," Mammary Gland Biology Gordon Research Conference, June 3-8, 2001, Bristol, RI.

CONCLUSIONS

The linked acquisitions of telomerase activity and TGFβ resistance observed in our HMEC system are likely to model key preneoplastic changes that occur during human breast cancer progression. We have begun to dissect the complex mechanisms responsible for these changes in expectation that such knowledge may enable us to prevent or reverse them. Our work thus far indicates that altered regulation of a well-known oncogene, c-myc, may link these processes by lowering the external signaling requirements for cell proliferation. It is not yet clear, however, how hTERT expression leads to altered c-myc regulation. The ability of the hTERT protein to cause TGFβ resistance does not appear to be separable from its ability to bind and lengthen telomeres in vivo. Finally, the ability of hTERT to cause TGFβ resistance does depend on cell context; only HMEC lacking p16 expression were susceptible to hTERT immortalization and TGFβ resistance. This latter finding suggests that particular cell types may be targeted in vivo.

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APPENDICES

Fig.1 C-myc mRNA shows aberrant regulation in fully immortal HMEC. The relative levels of c-myc mRNA were determined by Northern blot analysis for conditionally (184A1 23°) or fully immortal (184A1 46°) HMEC fed complete growth medium (cycling), arrested in G0 by blockage of EGF receptor signal transduction, or synchronized and refed with EGF for 1 hr. Note the higher c-myc mRNA levels in the fully immortal cells, particularly when both cultures were arrested synchronously in G0.

Fig. 2 C-myc protein shows aberrant regulation in hTERT-transduced HMEC. Total cell lysates were prepared from sub-confluent cultures of untreated finite lifespan HMEC (184@K 9p) or the same cells transduced with hTERT (184@K-TERT 14p and 33p) which were growth arrested in G0, synchronized in G0, then given back EGF for 1 hr., or randomly cycling. The immunoblot was probed with anti-c-myc antibodies, and signal detected by indirect chemoluminescence. Labeling index (LI) was determined by autoradiography of cells incubated with [^{3}H]thymidine for 24 hrs. prior to harvest.

Fig. 3 Tin2 expression delays the onset of TGFβ-resistant growth in conditionally immortal 184A1 cells. Growth capacity was assayed at indicated passages in single cell-derived colonies of 184A1 cells transduced with control vector (LXSN), Tin2, or TRF1 by autoradiography of cells incubated with [^{3}H] thymidine for 24 hrs. prior to harvest.

Fig. 4 Rare hTERT immortalization of preselection 184 HMEC is associated with gradual down-regulation of p16 and acquisition of TGFβ resistance. (A) Decreasing p16 expression (brown precipitate) in individual cells with increasing passage in the rare preselection HMEC that gained immortality after hTERT transduction at passage 3 [hTERT-184(3p)]. p16(-) postselection HMEC transduced with hTERT [184-TERT(11p)] are shown for comparison. (Bar = 100 μm.) (B) Western blot analysis showing decreasing p16 expression with increasing passage in hTERT-184(3p). (C) Increasing ability to grow in TGFβ correlates with decreasing p16 expression in hTERT-184(3p). LI was determined from five separate fields at the indicated days postseeding.

Fig.1 C-myc shows aberrant regulation in fully immortal HMEC.

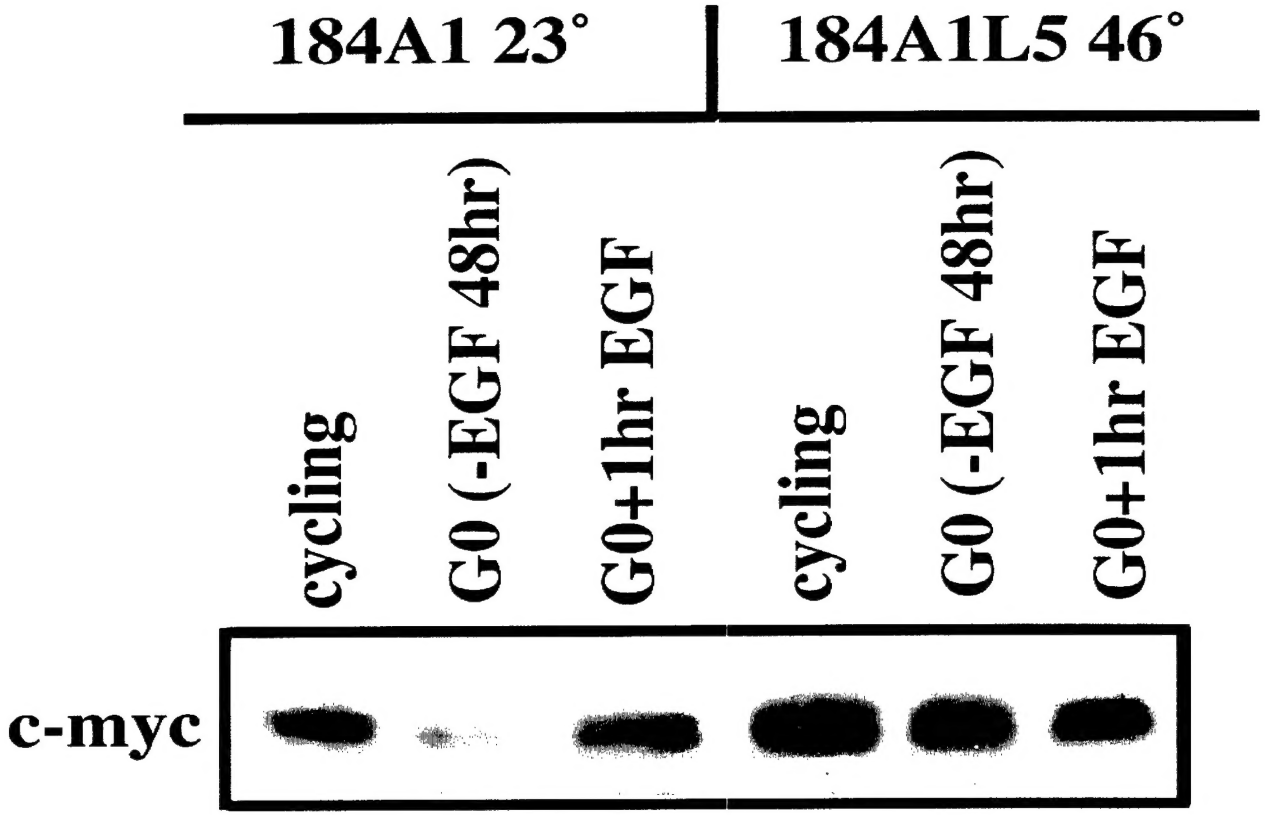

Fig. 2 C-myc regulation is altered in hTERT-transduced HMEC.

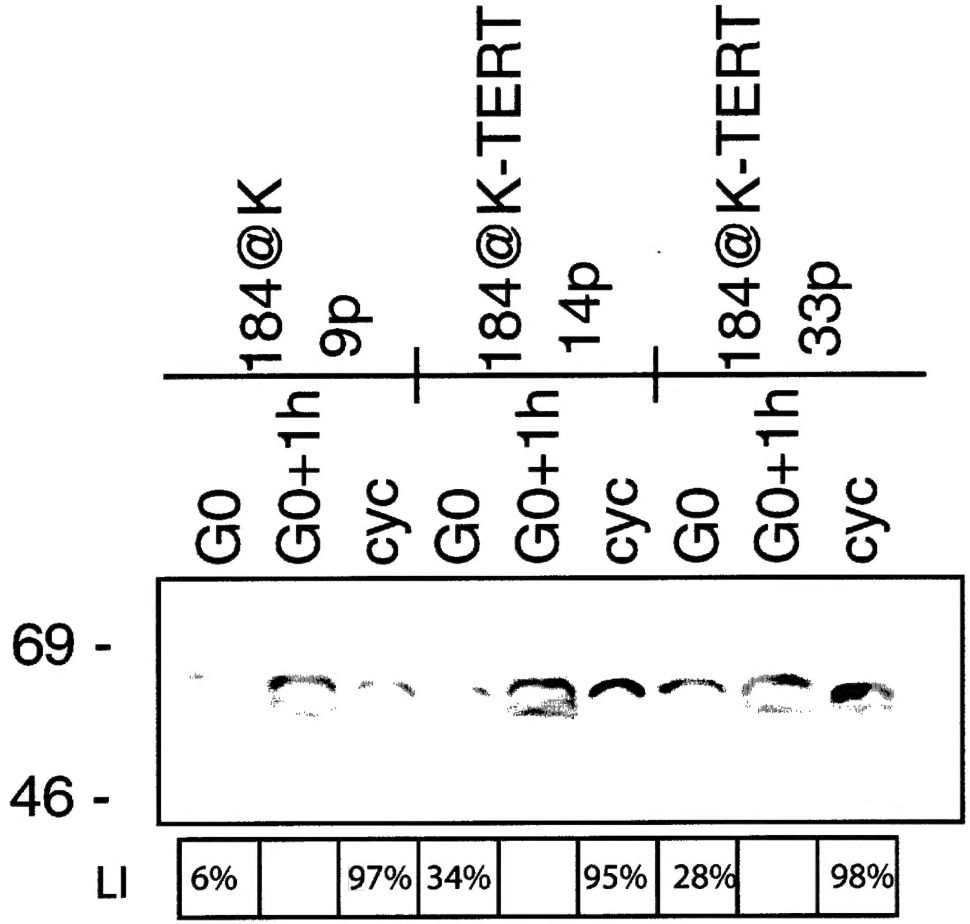

Fig.3 Conditionally immortal HMEC transduced with TIN2 display delayed acquisition of TGFβ resistance.

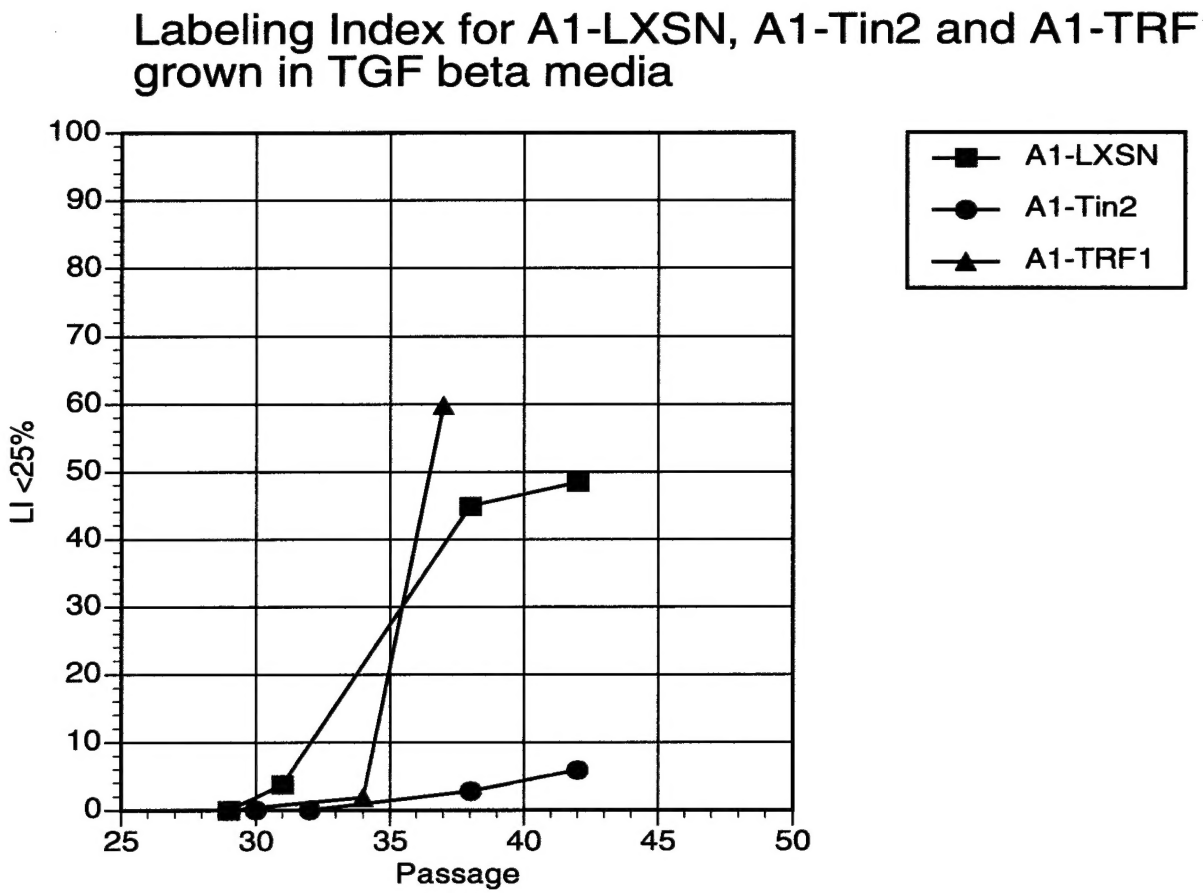

Fig.4 Rare hTERT immortalization of preselection 184 HMEC is associated with gradual down-regulation of p16 and acquisition of TGFβ resistance.